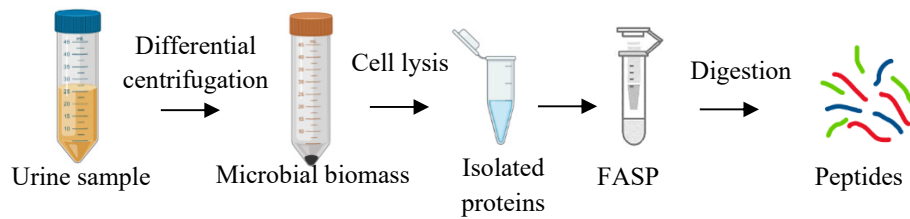


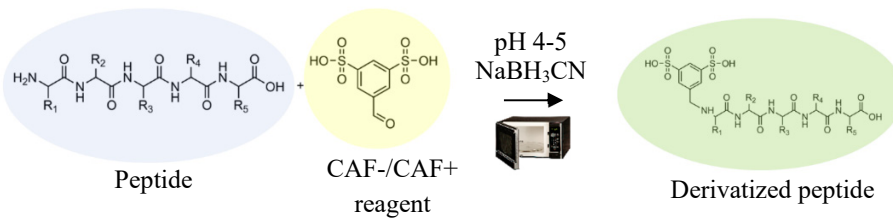
SAMPLE PREPARATION (Step 1)

Isolation of proteins from the urine sample and FASP trypsin digestion



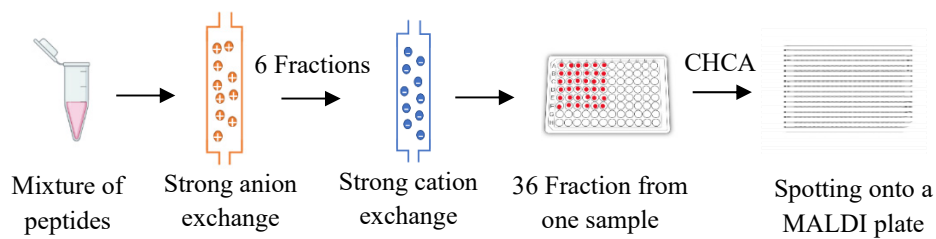
DERIVATIZATION (Step 2)

Microwave assisted derivatization of peptides



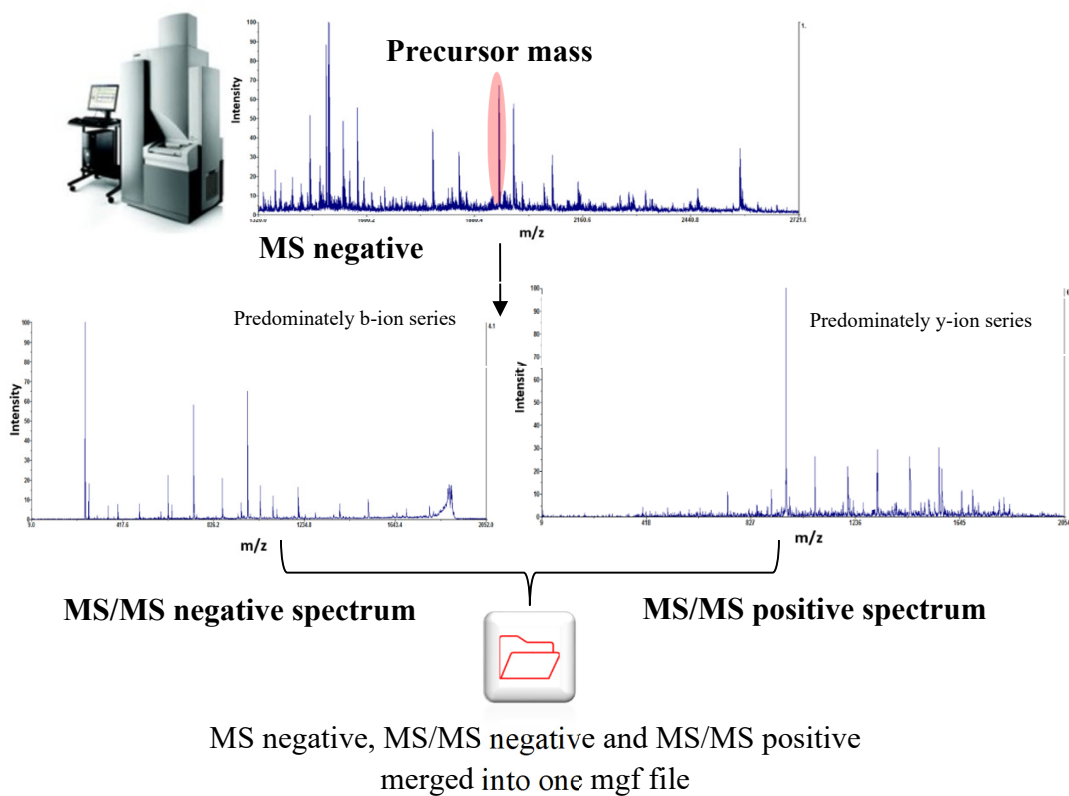
SEPARATION (Step 3)

2-D μSPE (SAX/SCX-36 fractions) automated AssayMap fractionation



ANALYSIS (Step 4)

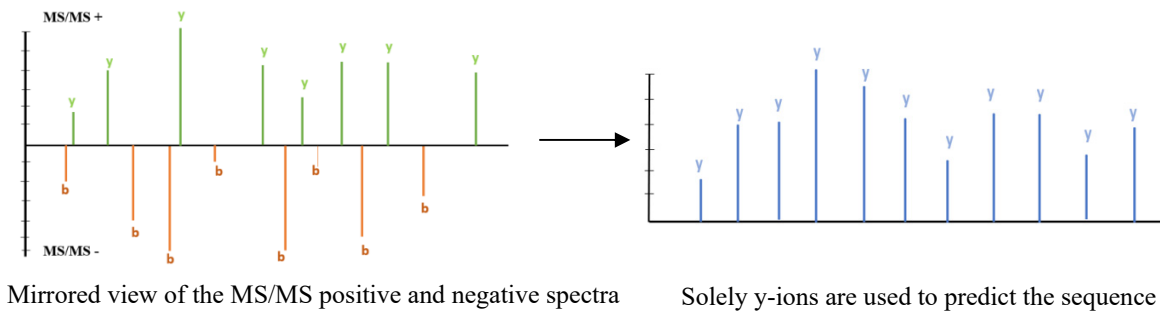
MALDI-TOF/TOF analysis MS negative (36 spots) followed by MS/MS negative/positive (720)
selected precursor masses in each mode)



IDENTIFICATION (Step 5)

Identification of microorganism by MALDI-MS/MS & *de novo* BLASTp using Protein Acrobat

Transformation of b-ions to y-ions
Precursor mass - b+1 = y



BLASTp

**Peptide to
Taxa match**

**Candidate
peptides**

Graph algorithm *de novo* sequencing

**Species
identification**

Decision tree to assess
identification confidence level

Figure S1. Workflow of five-step MALDI-MS/MS identification of urine microorganisms.

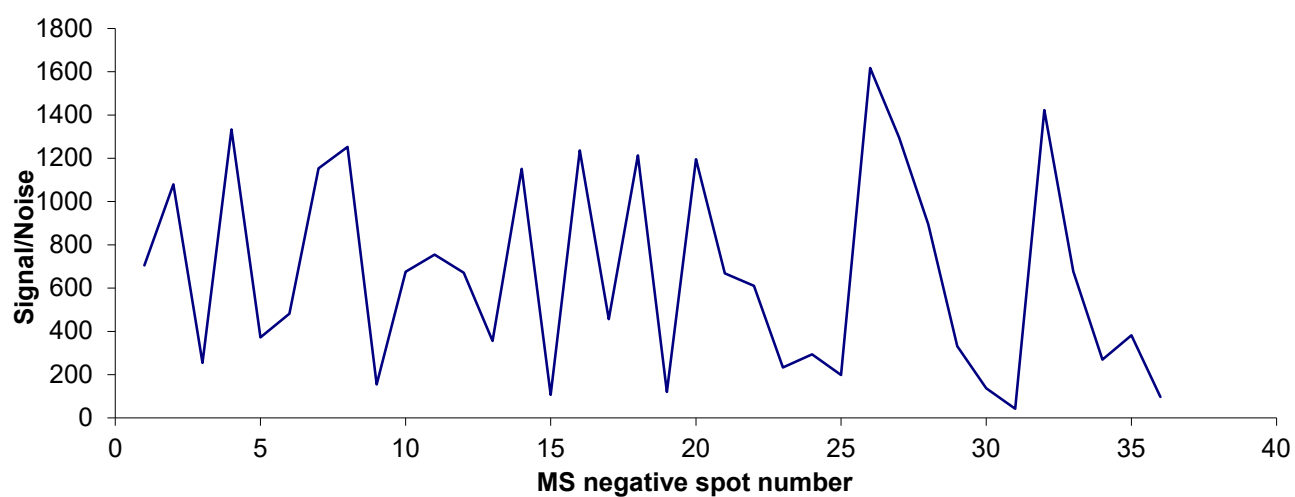


Figure S2. Mass spectra negative ion mode total signal-to-noise intensity per spot m/z 1000-3500.

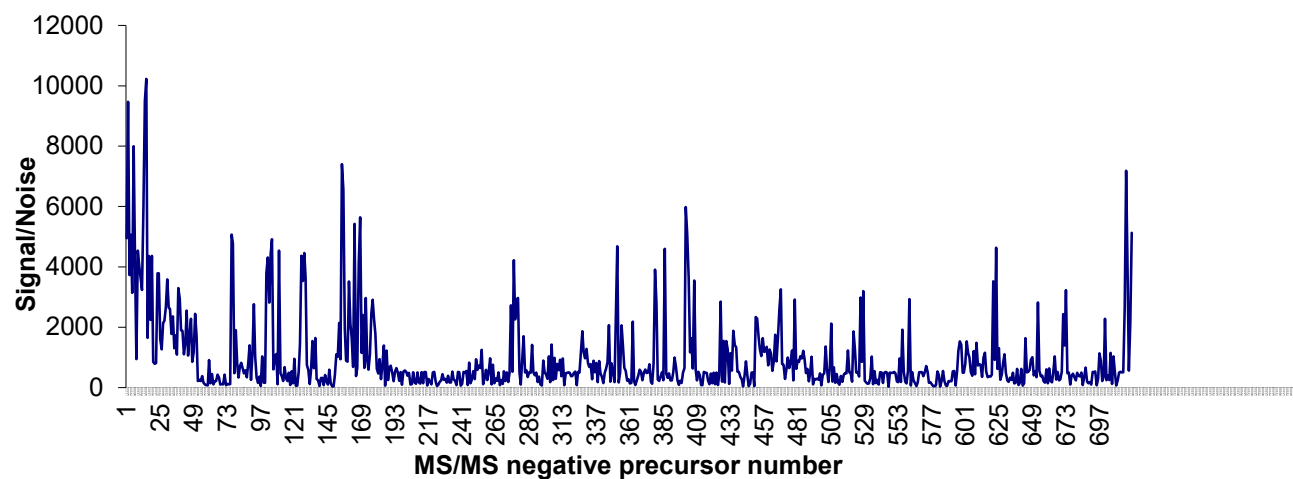


Figure S3. Tandem mass spectra negative ion mode total MS/MS signal-to-noise intensity per selected precursor ion spectrum.

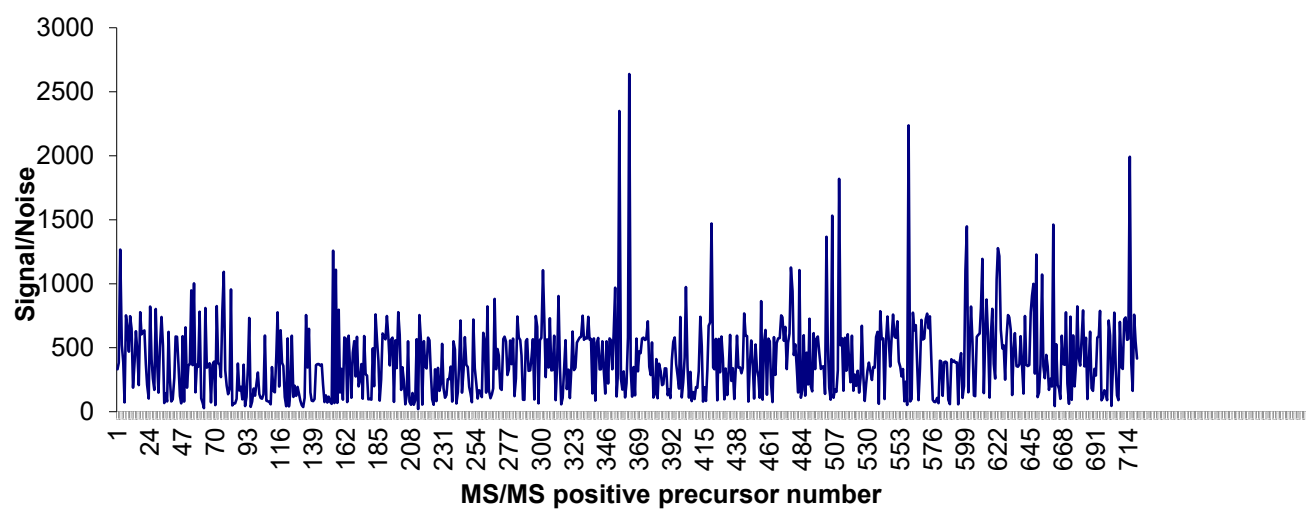


Figure S4. Tandem mass spectra positive ion mode total MS/MS signal-to-noise intensity per selected precursor ion spectrum.

Table S1: 48 common uropathogenic species adapted from Pinault et al. 2019 that were used to filter the NCBI*nr* database to enable the BLASTp search.

Number	name	preferred name	taxid
1	<i>Proteus mirabilis</i>		584
2	<i>Enterococcus faecalis</i>		1351
3	<i>Klebsiella pneumoniae</i>		573
4	<i>Enterobacter cloacae</i>		550
5	<i>Pseudomonas aeruginosa</i>		287
6	<i>Staphylococcus aureus</i>		1280
7	<i>Citrobacter koseri</i>		545
8	<i>Streptococcus agalactiae</i>		1311
9	<i>Staphylococcus saprophyticus</i>		29385
10	<i>Enterobacter aerogenes</i>	<i>Klebsiella aerogenes</i>	548
11	<i>Klebsiella oxytoca</i>		571
12	<i>Staphylococcus epidermidis</i>		1282
13	<i>Enterococcus faecium</i>		1352
14	<i>Morganella morganii</i>		582
15	<i>Citrobacter freundii</i>		546
16	<i>Serratia marcescens</i>		615
17	<i>Gardnerella vaginalis</i>		2702
18	<i>Proteus vulgaris</i>		585
19	<i>Stenotrophomonas maltophilia</i>		40324
20	<i>Streptococcus oralis</i>		1303
21	<i>Hafnia alvei</i>		569
22	<i>Providencia stuartii</i>		588
23	<i>Acinetobacter pittii</i>		48296
24	<i>Staphylococcus haemolyticus</i>		1283
25	<i>Pseudomonas putida</i>		303
26	<i>Staphylococcus hominis</i>		1290
27	<i>Enterobacter kobei</i>		208224
28	<i>Raoultella ornithinolytica</i>		54291
29	<i>Citrobacter amalonaticus</i>		35703
30	<i>Aerococcus urinae</i>		1376
31	<i>Acinetobacter baumannii</i>		470
32	<i>Enterococcus gallinarum</i>		1353
33	<i>Staphylococcus capitis</i>		29388
34	<i>Enterobacter asburiae</i>		61645
35	<i>Streptococcus anginosus</i>		1328
36	<i>Streptococcus mitis</i>		28037
37	<i>Streptococcus pneumoniae</i>		1313

38	<i>Acinetobacter nosocomialis</i>	106654
39	<i>Providencia rettgeri</i>	587
40	<i>Corynebacterium amycolatum</i>	43765
41	<i>Corynebacterium striatum</i>	43770
42	<i>Corynebacterium tuberculostrictum</i>	38304
43	<i>Pseudomonas plecoglossicida</i>	70775
44	<i>Enterobacter ludwigii</i>	299767
45	<i>Citrobacter braakii</i>	57706
46	<i>Pseudomonas mosselii</i>	78327
47	<i>Escherichia coli</i>	562
48	<i>Candida spp.</i>	1535326

Table S2: Buffers used for two-dimensional peptide fractionation by AssayMAP Bravo liquid handling platform. QMA= Quaternary methyl amin, ACN=Acetonitrile.

First dimension: Strong anion exchange	
Stationary phase	QMA
Priming buffer	400 mM NH ₄ HCOOH, 1% NH ₄ OH / 25% ACN
Equilibration buffer	1% NH ₄ OH
Elution buffer 1	80 mM NH ₄ CH ₃ COOH / 25% CH ₃ OH
Elution buffer 2	150 mM NH ₄ CH ₃ COOH / 25% CH ₃ OH
Elution buffer 3	200 mM NH ₄ CH ₃ COOH / 25% CH ₃ OH
Elution buffer 4	300 mM NH ₄ CH ₃ COOH / 25% CH ₃ OH
Elution buffer 5	500 mM NH ₄ CH ₃ COOH / 25% CH ₃ OH
Elution buffer 6	40 Mm NH ₄ HCOOH pH 3.5 / 25% CH ₃ OH
Second dimension: Strong Cation Exchange	
Stationary phase	SCX
Priming buffer	400 mM NH ₄ HCOOH, 1% HCOOH/ 25% ACN
Equilibration buffer	1% HCOOH / 25% ACN

Elution buffer 1	40 mM NH ₄ HCOOH, pH 3.5 / 25% ACN
Elution buffer 2	40 mM NH ₄ HCOOH, pH 4.0 / 25% ACN
Elution buffer 3	40 mM NH ₄ CH ₃ COOH, pH 4.5 / 25% ACN
Elution buffer 4	40 mM NH ₄ CH ₃ COOH, pH 5.0 / 25% ACN
Elution buffer 5	40 mM NH ₄ CH ₃ COOH, pH 6.0 / 25% ACN
Elution buffer 6	100 mM NH ₄ OH, pH 9.5 / 25% ACN